Effects of In Vitro Purging With 4-Hydroperoxycyclophosphamide on the Hematopoietic and Microenvironmental Elements of Human Bone Marrow

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We describe the effects of 4-hydroperoxycyclophosphamide (4-HC) on the hematopoietic and stromal elements of human bone marrow. Marrow cells were exposed to 4-HC and then assayed for mixed (CFU-Mix), erythroid (BFU-E), granulomonocytic (CFU-GM), and marrow fibroblast (CFU-F) colony-forming cells and studied in the long-term marrow culture (LTMC) system. The inhibition of colony formation by 4-HC was dose and cell-concentration dependent. The cell most sensitive to 4-HC was CFU-Mix (ID_{50} 31 μmol/L) followed by BFU-E (ID_{50} 41 μmol/L), CFU-GM (ID_{50} 89 μmol/L), and CFU-F (ID_{50} 235 μmol/L). In LTMC, a dose-related inhibition of CFU-GM production was noted. Marrows treated with 300 μmol/L 4-HC were completely depleted of CFU-GM but were able to generate these progenitors in LTMC. Marrow stromal progenitors giving rise to stromal layers in LTMC, although less sensitive to 4-HC cytotoxicity, were damaged by 4-HC also in a dose-related manner. Marrows treated with 4-HC up to 300 μmol/L, gave rise to stromal layers composed of fibroblasts, endothelial cells, adipocytes, and macrophages. Cocultivation experiments with freshly isolated autologous hematopoietic cells showed that stromal layers derived from 4-HC–treated marrows were capable of sustaining the long-term production of CFU-GM as well as controls. In conclusion: (1) Hematopoietic progenitors cells, CFU-Mix, BFU-E, and CFU-GM, are highly sensitive to 4-HC, whereas marrow stromal progenitor cells are relatively resistant. (2) Marrows treated with 300 μmol/L 4-HC that are depleted of CFU-Mix, BFU-E, and CFU-GM can generate CFU-GM in LTMC, suggesting that most primitive hematopoietic stem cells (not represented by CFU-Mix) are spared by 4-HC up to this dose. (3) Consequently, the above colony assays are not suitable tools for predicting pluripotent stem cell survival after 4-HC treatment in vitro.

4-HYDROPEROXYCYCLOPHOSPHAMIDE (4-HC) is a derivative of cyclophosphamide that exhibits in vitro chemical and biologic properties similar to those of microsomally activated cyclophosphamide.[1,2] In 1980, Sharkis et al[3] showed that 4-HC can selectively purge murine acute leukemia cells from marrow suspensions in a dose-related manner without affecting the viability and self-renewal capacity of the nonleukemic pluripotential hematopoietic stem cells (CFU-s). These studies provided the rationale for the application of 4-HC purged autologous bone marrow transplantation to the treatment of human leukemia[4] and lymphoma.[5] Thus autologous bone marrow harvested in remission, which has high risk of being contaminated with microscopically undetectable malignant cells, can be purged in vitro with 4-HC and then reinfused to reverse the lethal aplasia induced by preceding ablative chemoradiotherapy.[4,5]

The successful engraftment of transplanted bone marrow requires, besides intact primitive hematopoietic stem cells, a functional microenvironment or stroma in which hematopoietic stem cells can self-replicate and differentiate.[6,7] In humans, the marrow stromal cell (MSC) population comprises fibroblasts, endothelial cells, adipocytes, and macrophages.[8-10] Recent studies have shown that the stromal cells forming the in vitro microenvironment in human Dexter-type long-term marrow culture (LTMC) generated from marrow aspirates of allogeneic marrow transplant recipients are of donor origin and that the percentage of donor cells contributing to the culture stromal microenvironment progressively increases in marrow aspirates taken at greater times after transplantation.[11] Although no evidence has been provided that the proliferation of donor MSCs in vivo is necessary to support grafted hematopoietic cells, it is conceivable that transplantation of MSCs constitutes a factor of critical functional significance.[12]

The studies presented here were aimed at evaluating the effects of 4-HC on the hematopoietic stem cells and stromal elements of human bone marrow. Evidence is presented demonstrating that multipotential (CFU-Mix) and erythroid (BFU-E) colony-forming cells are highly sensitive to the cytotoxic effect of 4-HC. In contrast, MSCs are relatively resistant and not functionally affected by the doses of 4-HC currently used for purging autologous bone marrow.

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MATERIALS AND METHODS

Bone Marrow Cells

Bone marrow cells were obtained by aspiration from the iliac crest of healthy volunteers who gave informed written consent. Preservation-free heparin was used as anti-coagulant (Weddel Pharmaceuticals Ltd, London). Buffy coats were collected after centrifugation of the aspirates at 200 g for ten minutes. Cells were washed and resuspended in alpha modification of Eagle's medium (Flow Laboratories, Hamden, Conn) supplemented with 10% fetal calf serum ([FCS] Sterile Systems, Logan, Utah).

4-Hydroperoxycyclophosphamide and Incubation Procedure

The 4-HC (mol wt 292) used in the present experiments was prepared by Dr Michael O. Colvin (from the Johns Hopkins Oncology Center, Baltimore). The synthesis and purification procedures have been described elsewhere.[1] The 4-HC powder was dissolved in calcium- and magnesium-free phosphate-buffered solution (PBS), then sterilized by filtration, and used within 30 minutes.

Marrow buffy coat cells at a final concentration of 10 or 20 $\times 10^6$ cells per milliliter were incubated at 37 °C for 30 minutes with 25 to 500 μmol/L 4-HC and medium control. The cell suspensions were then washed at 10 °C and assayed for colony-forming cells or inoculated for long-term culture according to the experimental design. The incubation procedure was similar to the one used for clinical trials of 4-HC–purged autologous marrow transplantation. Ten percent FCS was constantly included in all marrow suspensions. Viability was not significantly changed by the incubation procedure.

Colony Assays

Mixed and erythroid colony-forming cells. The assay for CFU-Mix was carried out according to the method of Fauser and Messner[13] as described previously.[14] Control and 4-HC–treated bone marrow cells were plated at 2×10^5 in 35-mm tissue culture dishes (Lux Scientific Co, Newburg, Calif) containing a 1-mL mixture of Iscove's modified Dulbecco medium, 1% methyl-cellulose, 30% FCS, 5% medium conditioned by leukocytes in the presence of 1% phytohemagglutinin (HA-15, Wellcome Reagents Ltd, Wellcome Research Laboratories, Detroit) and 5×10^{-5} mol/L 2-mercaptoethanol and 1 unit of a step III preparation of sheep plasma erythropoietin (Connaught Laboratories Ltd, Willowdale, Ontario, Canada). Dishes were incubated at 37 °C in a humidified atmosphere flushed with 5% CO_2 in air. CFU-Mix were scored with an inverted microscope after 14 days of incubation and were identified further by plucking out colonies with a fine pipette and staining with benzidine and/or Wright-Giemsa stain. CFU-Mix usually contained erythroid, granulocyte, monocytic, and megakaryocytic cells. BFU-E were scored from the same plates.

Granulocyte-macrophage colony-forming cells. Colony (more than 50 cells per aggregate) and cluster (three to 50 cells per aggregate) formation of control and 4-HC–treated bone marrow cells were stimulated by 10% exogenously supplied granulocyte-macrophage colony-stimulatory factors present in medium conditioned by the human monocytic cell line GCT (GIBCO Laboratories, Grand Island, NY). Control and 4-HC–treated cells were plated at 2×10^5 in 1 mL of 0.3% agar culture medium (Difco Labs, Detroit) that included McCoy's 5A medium supplemented with essential and nonessential amino acids, glutamine, serine, asparagine, sodium pyruvate (GIBCO) as well as 10% heat-inactivated fetal bovine serum. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air and scored for colonies and clusters after seven days of incubation.

Fibroblast colony-forming cells. The general procedure has been described previously.[15,16] Briefly, control and 4-HC–treated marrow cells resuspended in alpha-medium supplemented with 20% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL) were cultured in T-75 tissue culture flasks (Corning Glass Works, Corning, NY). Five $\times 10^6$ cells were incubated per flask; three flasks were used per point. The flasks were gassed with 5% CO_2 in air and incubated at 37 °C. The culture medium was totally renewed on day 4. After ten days of incubation, the cultures were stopped. For scoring fibroblast colonies, the flasks were stained with Wright-Giemsa stain and examined with an inverted microscope at 25×. Fibroblastoid cell aggregates of more than 50 cells were scored as CFU-F. The fibroblastic nature of cells composing the colonies was demonstrated by immunofluorescence staining with antibodies against fibronectin and types I and III collagen as described previously.[15,16]

Long-term Marrow Cultures

LTMCs were established according to the method of Moore et al[17,18] as modified by Gartner and Kaplan.[19] Twenty to forty $\times 10^6$ control and 100, 300, and 400 μmol/L 4-HC–treated marrow buffy coat cells in 10 mL of LTMC medium were inoculated into T-25 tissue culture flasks. The cultures were then gassed with 5% CO_2 in air and incubated at 33 °C. The LTMC medium consisted of McCoy's 5A medium supplemented with 1% minimal essential medium (MEM) sodium pyruvate solution, 0.8% MEM essential amino acids solution, 0.4% MEM nonessential amino acids solution, 1% MEM vitamins solution, 1% penicillin-streptomycin solution, 1% glutamine-asparagine-serine solution, 12.5% horse serum, 12.5% FCS, and 10^{-6} mol/L hydrocortisone sodium succinate. On day 3 of incubation, in order to eliminate the contaminating erythrocytes and mature myeloid cells, the nonadherent cells were gently aspirated out of the flasks and enriched for mononuclear cells by neutral density centrifugation in an isotonic sterile Percoll solution (1.074 g/mL, 270 mosm) (Pharmacia Fine Chemicals, Piscataway, NJ) as described previously.[20] The resulting buoyant, mononuclear cell–enriched fraction was collected, washed twice, resuspended in 5 mL of LTMC medium, and reinoculated into the original flasks containing the adherent stromal layers and fresh LTMC medium. The latter was added (5 mL per flask) soon after the nonadherent cells were aspirated out of the flasks. At weekly intervals, half the supernatant medium and suspension cells were removed and replaced with fresh LTMC medium. Cells in suspension were counted, checked for viability by trypan blue dye exclusion test, and assayed for CFU-GM. Individual LTMCs were examined every seven days under a phase contrast inverted microscope, and the percentage of the flask surface covered by a stromal interlocking network was semiquantitatively assessed. Grades 1 through 4 corresponded to 25% to 100% of the base of each flask that was covered by a stromal network. At the time of stopping the culture (week 6 to 7), the CFU-GM content of the adherent layers derived from control and 4-HC–treated marrows was assessed using the following procedure. Individual flasks were gently washed twice to remove remaining nonadherent cells, and 2 mL of a 0.01% (wt/vol) Ca^{++}-Mg^{++}–free trypsin (GIBCO) solution was added to the flask and it was incubated at 4 °C for 15 to 20 minutes. The cells that had been detached by the trypsin treatment (80% to 90% of the adherent fraction) were aspirated out from the flask, diluted 1:1 with McCoy's medium containing 10% FCS, counted, and assayed for CFU-GM as the nonadherent cell fraction. In independent experiments using fresh marrow buffy coat cells, this trypsin treatment inhibited the CFU-GM growth by less than 5%.

To assess the capacity of the stromal layers established from 100

μmol/L 4-HC–treated marrows to sustain hematopoiesis in vitro, five-week-old primary LTMCs were totally depleted of all suspension cells, and a second inoculum of autologous bone marrow was added. This consisted of 20 × 10^6 light-density (<1.074 g/mL) Percoll-separated nonadherent autologous marrow cells. Percoll-separated cells were allowed to adhere to the bottom of T-75 tissue culture flasks at 37 °C twice, for one hour each time. After adherence, the nonadherent light-density (NAL) autologous cells were extensively washed and added as dispersed single-cell suspension in fresh LTMC medium. The experimental groups included (a) NAL cells on stromal layers from 4-HC–treated marrow, (b) cell-free LTMC medium on stromal layers from 4-HC–treated marrow, (c) NAL cells in LTMC medium without any stromal layer, (d) NAL cells on control stromal layers, and (e) cell-free LTMC medium on control stromal layers. Cultures were demipopulated weekly and fed with the same amount of fresh LTMC medium. The total cell counts and CFU-GM numbers per flask were determined weekly.

Immunocytochemical Analysis of the Adherent Layer of LTMCs

The heterogeneity of the cells constituting the adherent layer of LTMCs established with 4-HC–treated marrow was assessed by immunofluorescence (IMF) methods using specific antibodies directed against components known to be associated with fibroblasts, endothelial cells, and macrophages as described previously.[15,16,21] These studies were performed in situ after the removal of the upper portion of culture flasks with a heated scalpel and double washing with PBS, pH 7.4.

Fibroblasts were identified by rabbit antibodies to type III collagen (kindly provided by Dr S. Gay from the University of Alabama) and human fibronectin (Bethesda Research Laboratories, Gaithersburg, Md). Endothelial cells were identified by rabbit antiserum to human factor VIII–related protein (Calbiochem-Behring, San Diego). Macrophages were identified by mouse monoclonal antibodies to human monocytes (Bethesda Research Labs). Furthermore, the distribution of lipid-containing cells was studied using the oil red O staining for neutral fat as elsewhere described.[22]

RESULTS

Effect of 4-Hydroperoxycyclophosphamide on Multipotential, Erythroid, and Granulocyte-Macrophage Colony-Forming Cells

The percentage of recovery of CFU-Mix, BFU-E, and CFU-GM in comparison to that of CFU-F is depicted in Fig 1. Treatment of marrow buffy coat cells at 20 × 10^6 cells per milliliter with 100 μmol/L 4-HC resulted in 100% ± 0%, 97% ± 1%, 71% ± 6%, and 17% ± 7% inhibition of CFU-Mix, BFU-E, CFU-GM, and CFU-F formation, respectively. Treatment with doses higher than those indicated in Fig 1, ie, 150, 200, 300, and 500 μmol/L 4-HC, resulted in 80% ± 2%, 96% ± 2%, 99.0% ± 0.5%, and 100% ± 0% inhibition of CFU-GM growth. The ID_{50} of CFU-Mix, BFU-E, and CFU-GM formation was 31, 41, and 89 μmol/L 4-HC.

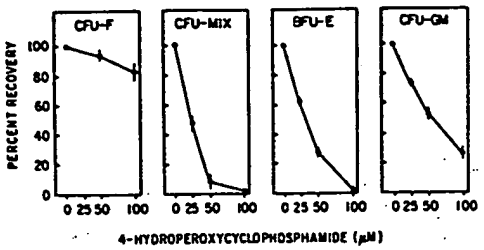

Fig 1. Comparison of the effect of 4-HC on human marrow stromal and hematopoietic progenitor cells. Marrow cells were incubated with 4-HC, washed, and then assayed for fibroblast (CFU-F), mixed (CFU-Mix), erythroid (BFU-E), and granulocyte-macrophage (CFU-GM) colony-forming cells. Values shown are the mean ± SEM from four (CFU-GM) and three (CFU-Mix, BFU-E) separate experiments and are expressed as percentage of control values. The control for CFU-Mix was 10.3 ± 0.9; BFU-E, 98 ± 26; CFU-GM, 79 ± 23; CFU-F, 91 ± 11. Note that CFU-F are relatively resistant to 4-HC as compared with CFU-Mix, BFU-E, and CFU-GM.

Treatment of marrow buffy coat cells at a lower cell concentration, ie, 10 × 10^6 per milliliter, with 50, 100, 150, 200, 300, and 500 μmol/L 4-HC resulted in 59% ± 19%, 87% ± 5%, 96% ± 2%, 99.0% ± 0.5%, 100% ± 0%, and 100% ± 0% inhibition of CFU-GM growth, respectively. The ID_{50} of CFU-GM formation was 22 μmol/L 4-HC.

When 25 μmol/L 4-HC–treated marrow cells were mixed at different ratios with autologous untreated marrow buffy coat cells and plated for CFU-GM, BFU-E, and CFU-Mix, the observed number of colonies closely corresponded to the expected values calculated on the basis of dilution in the cell mixtures (Table 1).

Effect of 4-Hydroperoxycyclophosphamide on Marrow Fibroblast Colony-Forming Cells

The percentage of recovery of CFU-F after incubation with 50 to 500 μmol/L 4-HC is shown in Fig 2. The pattern of recovery was clearly dose and cell-concentration dependent. The dose of 4-HC inhibiting 50% (ID_{50}) of the growth of CFU-F was 235 μmol/L

Table 1. Influence of Untreated Marrow Cells on CFU-GM, BFU-E, and CFU-Mix Growth From 4-HC–Treated Human Bone Marrow

4-HC–Treated-Untreated Ratio of Cultured Cells	CFU-GM per 10^5 Cells			BFU-E per 2 × 10^5 Cells			CFU-Mix per 5 × 10^5 Cells		
	Observed	Expected	O/E	Observed	Expected	O/E	Observed	Expected	O/E
3:0	27.2	—	—	55	—	—	3.5	—	—
2:1	39.9	41.5	0.96	60	58.2	1.03	4.0	5.0	0.80
1:1	51.0	48.7	1.04	63	60.0	1.05	6.5	6.0	1.08
1:2	53.7	55.8	0.96	66	61.6	1.07	6.1	6.7	0.91
0:3	70.2	—	—	65	—	—	8.5	—	—

O/E, observed-expected ratio.

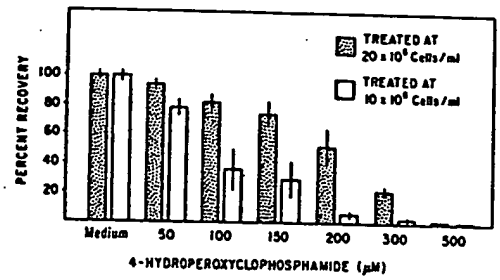

Fig 2. Effect of 4-HC on human marrow fibroblast colony-forming cells. Marrow cells were incubated with 4-HC, washed, and then assayed for CFU-F. Values shown are the mean ± SEM from five separate experiments and are expressed as percentage of control values. The control CFU-F was 91 ± 11. Note that the toxic effect of 4-HC is dose and cell-concentration dependent.

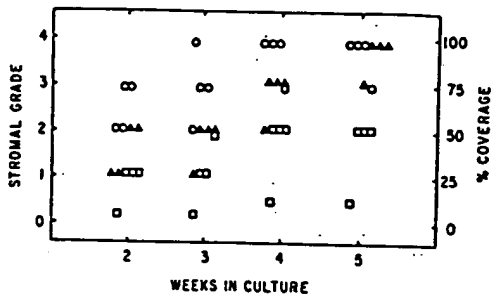

Fig 3. Stromal development in long-term cultures of 4-HC–treated human bone marrow. Marrow cells (20×10^6 per milliliter) were incubated with 100 μmol/L 4-HC, washed, and then inoculated for long-term culture. At weekly intervals, the stromal layer from experimental groups was graded by phase microscopy as described in Materials and Methods. Results are from four separate experiments, and each point represents the median score of triplicates. A heterogeneous stromal layer can be established after treatment with 4-HC, although a double number of 4-HC–treated cells is required to obtain a stromal layer similar to controls. O, Control: 20×10^6 cells/flask; □, 4-HC treated: 20×10^6 cells/flask; ▲, 4-HC treated: 40×10^6 cells/flask.

when the cells were treated at a final concentration of 20×10^6 cells per milliliter. The ID_{50} was 115 μmol/L when the incubation with 4-HC was carried out at 10×10^6 cells per milliliter. Thus treatment at a higher cell concentration resulted in a lower cytotoxic effect of 4-HC on CFU-F.

Establishment of LTMCs With 4-Hydroperoxycyclophosphamide–Treated Bone Marrow

Human bone marrows treated with 100 and 300 μmol/L 4-HC and then cultured in LTMC were ultimately capable of giving rise to an adherent stromal layer. In contrast, treatment of the same marrows with 400 μmol/L 4-HC impaired their capacity to establish an LTMC. Figure 3 shows the stromal development in cultures of control and 100 μmol/L 4-HC–treated marrow as a percentage of the surface of the culture flask covered by the stromal network. At initial stages of culture, weeks 1 to 3, the extent of the stromal layer derived from untreated marrow was more extensive. However, in the following weeks, when confluence was already reached in the untreated group, the 4-HC–treated stromal layers became progressively confluent and comparable to controls. Inoculation of a higher number of treated marrow cells (40×10^6 per flask) resulted in the formation of stromal layers equivalent in extent to those formed by 20×10^6 untreated marrow cells.

The analysis of the distribution and identify of the cells composing the stromal layer of five-week-old LTMCs established with 4-HC–treated marrows is shown in Table 2. In LTMCs derived from 100 and 300 μmol/L 4-HC–treated marrows, fibroblasts constituted the predominant (>75%) adherent cell population. Endothelial cells were found sparsely (<10%) distributed within these stromal layers, except in one experiment among the 300 μmol/L 4-HC–treated group. Groups of lipid-containing cells were present in all cultures. Ten percent to 25% (three experiments) and 40% (one experiment) macrophages could be identified in the stromal layers derived from 100 μmol/L 4-HC–treated marrows. Less than 10% (two experiments) and 10% to 25% (one experiment) macrophages were found in the stromal layers derived from 300 μmol/L 4-HC–treated marrows. In contrast, the in vitro stroma derived from 400 μmol/L

Table 2. Extent and Composition of the Stromal Layers of Five-Week-Old Long-Term Cultures Derived From 4-HC–Treated Human Bone Marrow

Experiment No.	4-HC 100 μmol/L					4-HC 300 μmol/L					4-HC 400 μmol/L				
	Cvrg	F	EC	A	M	Cvrg	F	EC	A	M	Cvrg	F	EC	A	M
1	100	++++	+	++	++	75	++++	+	++	++	<10	±	ND	—	—
2	100	+++	+	++	+++	75	++++	—	++	+	<10	±	—	—	—
3	75	++++	+	++	++	100	++++	+	++	+	<10	—	ND	—	—
4	100	++++	+	++	++										

A heterogeneous stromal layer derived from 20×10^6 untreated buffy-coat cells exhibits the following composition: fibroblasts, 3^+ to 4^+; endothelial cells, ± to 1^+; adipocytes, 2^- to 3^+; macrophages 2^+ to 3^+. All cultures were initiated with 40×10^6 4-HC–treated marrow buffy coat cells per flask.

Cvrg, percentage of coverage of culture flask surface; F, fibroblast; EC, endothelial cell; A, adipocyte; M, macrophage; ND, not determined; —, 0%; +, <10%; ++, 10% to 25%; +++, 26% to 75%; ++++, >75%.

4-HC–treated marrows consisted only of a few scattered spots of fibroblasts (Table 2).

To assess the functional capacity of stromal layers established from 4-HC–treated marrow to sustain hematopoiesis in vitro, primary LTMCs were totally depleted of all suspension cells after five weeks, when the total CFU-GM produced per culture was reduced to 45 ± 21. Five groups of cultures were then initiated using a second addition of autologous NAL marrow cells (see Materials and Methods). As shown in Fig 4, no impairment in the production of CFU-GM was seen in the group involving the coculture of NAL cells with stromal layers from 4-HC–treated marrow as compared with stromal layers derived from untreated marrow. In flasks inoculated with NAL cells without any stromal support, the production of CFU-GM was remarkably lower and of short duration (Fig 4). In no instance were CFU-GM found in the nonadherent fraction of the flasks depopulated of all buoyant cells and not reinoculated with fresh NAL cells (not shown in Fig 4). This finding ruled out the possibility that control number of CFU-GM were released from the adherent layers derived from 4-HC–treated marrow.

The numbers of total CFU-GM and nonadherent cells in primary long-term cultures derived from control and 4-HC–treated marrows are summarized in Table 3. In comparison to control cultures, in the first two weeks the decline of the total nonadherent cell number was slower in the 100 and 300 μmol/L 4-HC–treated groups. Despite weekly demipopulation of the cultures, continuous production of CFU-GM could be detected in the nonadherent fraction of the 100 μmol/L 4-HC–treated group for five weeks. In the 300 μmol/L 4-HC–treated group, although the primary inoculum was virtually depleted of all CFU-GM (0.16 ± 0.20 per culture), a modest generation of these committed stem cells was found on week 1 and week 2 of culture. Treatment with 400 μmol/L 4-HC abolished CFU-GM growth in LTMCs. On weeks 6 to 7 of culture, CFU-GM were occasionally found in the adherent fraction of control LTMC, whereas in no instance could CFU-GM be detected in the LTMC derived from 4-HC–treated marrows.

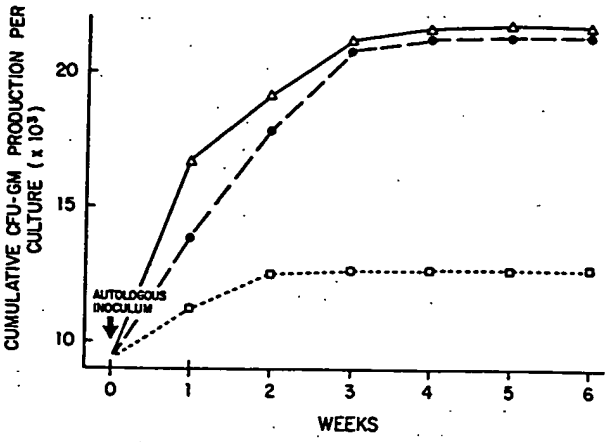

Fig 4. Sustained production of granulocyte-macrophage colony-forming cells on heterogeneous stromal layers derived from 4-HC–treated marrow. After depopulation of all suspension cells, 20 × 10⁶ autologous NAL marrow cells were added to five-week-old stromal layers of long-term cultures initiated with 40 × 10⁶ 4-HC–treated cells per flask. Cultures were demipopulated weekly, and the total cell counts and CFU-GM numbers per flask were determined. Each datum point represents the cumulative number of mean CFU-GM recovered from the cultures (two to three culture flasks per group). No correction was made for the depopulations owing to the weekly replacement of half the LTMC medium volume. In this representative experiment, the NAL cell inoculum containing 9.3 × 10³ CFU-GM was added to 4-HC–pretreated stromal layers (●), non-pretreated stromal layers (△), and controls with no stromal layers (□). Heterogeneous stromal layers derived from 4-HC–treated marrow could sustain the long-term production of CFU-GM in a manner similar to controls. No CFU-GM growth was observed in additional controls with no added cells: layers from 4-HC–treated marrow and layers from non-pretreated marrow (data not shown in this figure).

DISCUSSION

Data presented in this paper show that the 4-HC doses currently used for marrow purging in autologous transplantation, although depleting the graft of hematopoietic progenitors, including the putative pluripo-

Table 3. Primary Long-term Cultures Derived From Control and 4-HC–Treated Human Bone Marrow

Week	Total Nonadherent Cells per Culture (× 10⁶)				Total CFU-GM per Culture			
	Medium Control†	4-HC (μmol/L)‡			Medium Control†	4-HC (μmol/L)‡		
		100	300	400		100	300	400
1	91 ± 29	76 ± 19 (41)	25 ± 20 (14)	17 ± 10	4,133 ± 275	1,312 ± 387 (16)	13 ± 7 (<1)	0
2	34 ± 11	36 ± 23 (52)	21 ± 13 (31)	1.3 ± 1.0	1,828 ± 321	364 ± 98 (10)	1.1 ± 0.5 (<1)	0
3	10 ± 7	6.6 ± 1.5 (33)	1.5 ± 1.0 (7)	0.3 ± 0.1	370 ± 21	116 ± 36 (16)	0	0
4	6 ± 1	3.6 ± 3.0 (30)	0.8 ± 0.5 (7)	0.3 ± 0.1	159 ± 87	38.3 ± 19.0 (12)	0	0
5	2 ± 1	0.6 ± 0.5 (15)	0	0	45 ± 21	3.3 ± 3.0 (4)	ND	ND
6	0.6 ± 0.5	0	0	0	3.3 ± 3.0	0	ND	ND

Mean ± SEM of four (100 μmol/L 4-HC) and three (300 and 400 μmol/L 4-HC) separate experiments. All cultures were incubated at 33 °C and subjected to removal of half the growth medium and nonadherent cells at weekly intervals. †, primary inoculum 20 × 10⁶/per flask containing 9,164 ± 497 CFU-GM; ‡, primary inoculum 40 × 10⁶/per flask containing 2,610 ± 301 (100 μmol/L 4-HC group), 0.16 ± 0.20 (300 μmol/L group), and 0 (400 μmol/L group) CFU-GM; ( ), percentage of control cultures corrected for the number of initial inoculum; ND, not determined.

tent stem cells or CFU-Mix, do not significantly affect the number and function of MSC. The dose of 4-HC used in clinical marrow purging is limited by its toxicity on hematopoietic stem cells. This has been estimated indirectly by assaying the frequency of CFU-GM in the harvested marrows before and after 4-HC treatment.[23] Interestingly, clinical transplantation data have shown that despite CFU-GM depletion, 4-HC–purged autologous grafts retain their capacity to reconstitute the hematopoietic system of transplant patients pretreated with myeloablative chemoradiotherapy.[4,5] This lack of correlation between in vitro CFU-GM recovery and in vivo marrow-repopulating ability suggests that (1) the measured CFU-GM does not reflect the survival of primitive pluripotential stem cells, or (2) the treatment with 4-HC causes the loss of an accessory cell(s) necessary for the in vitro but not for the in vivo growth of hematopoietic stem cells. To address the first possibility, we studied the 4-HC sensitivity of CFU-Mix, a progenitor cell with self-renewal characteristics[24–26] that has been considered the putative primitive stem cell. Our results demonstrate that CFU-Mix and BFU-E are even more 4-HC–sensitive than is CFU-GM. The possibility that 4-HC is toxic to an accessory cell(s) necessary for the in vitro growth of hematopoietic stem cells was ruled out by the results of mixing experiments (4-HC–treated + untreated marrow cells at various ratios). Therefore, the CFU-Mix assay also appears to be an unsuitable tool for predicting the engraftment capability of 4-HC–purged grafts. Moreover, the fact that 100 and 300 μmol/L 4-HC–treated bone marrow (ie, depleted of CFU-Mix) can reinstitute full hematopoietic function in supralethally irradiated patients[4,5] indicates that CFU-Mix does not represent the stem cell responsible for hematopoietic reconstitution of the transplanted host and suggests that there are early pluripotent stem cells that are significantly less affected by 4-HC.

In an attempt to shed further light on the sensitivity of early hematopoietic stem cells, we measured the production of CFU-GM in primary LTMCs derived from control and 4-HC–treated marrow. Despite weekly demipopulation of the cultures, CFU-GM could be detected in the nonadherent fraction up to week 5 (100 μmol/L 4-HC–treated group) and week 2 (300 μmol/L 4-HC–treated group) of culture. In the latter group, a low number of CFU-GM was generated despite almost complete CFU-GM depletion in the initial inoculum. In contrast, 400 μmol/L 4-HC–treated marrows failed in all instances to generate any CFU-GM. These data suggest that the primitive pluripotent stem cells are affected by 4-HC also in a dose-related manner but to a lesser degree than the more differentiated hematopoietic stem cells, since their capacity to proliferate and differentiate into CFU-GM is spared by treatment with 4-HC up to 300 μmol/L. Two lines of evidence support this notion. First, previous studies by our group have shown that the probable human pluripotent stem cells that are detected in LTMC are Ia-antigen–negative,[18] whereas human CFU-Mix are Ia-antigen–positive.[14] This difference suggests that the two assays detect distinct cells within a hierarchy of stem cell differentiation, proliferation, and self-renewal capacity. Second, Botnick et al[27] in mice and Smith et al[28] in humans have shown that very-high-dose cyclophosphamide administration in vivo results in rapid depletion of committed hematopoietic stem cells and pancytopenia, which is followed by prompt hematologic recovery. These findings imply that cyclophosphamide or derivatives are more sparing of the most primitive hematopoietic stem cells.

Assessment of the effects of 4-HC on the MSC compartment by the LTMC system and the CFU-F assay showed that human MSCs are quantitatively but not functionally affected by the in vitro procedures for marrow purging with 4-HC. The unique adherent stromal layer found in the LTMC system is deemed necessary for continuing in vitro hematopoiesis and is the closest laboratory equivalent to its in vivo counterpart. In that marrow fibroblasts constitute the predominant cell population of MSCs and appear to play a significant role in regulation and differentiation of hematopoietic stem cells,[29–33] we used the CFU-F assay to quantitate the changes occurring on marrow fibroblast progenitors after treatment with 4-HC. In contrast to hematopoietic progenitors, MSCs are relatively resistant to the in vitro action of 4-HC. In fact, much higher 4-HC doses (CFU-F ID_{50} 235 μmol/L 4-HC *v* CFU-Mix ID_{50} 31 μmol/L 4-HC) are required to abolish the formation of colonies by CFU-F as well as the capacity of 4-HC–treated marrow suspensions to give rise to functional and heterogeneous stromal layers in LTMC. Furthermore, because the 4-HC cytotoxicity was dose dependent on CFU-F as well as on the stromal progenitors giving rise to LTMC adherent layers, it is possible that the CFU-F assay might reflect, as well as the frequency of fibroblasts, the frequency of the other components of the marrow stromal population in the bone marrow graft. In addition to measuring the direct toxic effect of 4-HC on MSCs, we tested the functional capacity of stromal layers derived from 4-HC–treated marrows by assessing their capacity to support long-term hematopoiesis. Such stromal layers did indeed support the long-term production of CFU-GM in a manner similar to controls, indicating that 4-HC treatment spares enough

MSCs to be capable of giving rise to heterogeneous stromal layers having a normal hematopoietic functional activity. Hilton has recently suggested, on the basis of studies done on human and rodent leukemia cell lines, that the intracellular aldehyde dehydrogenase (AHD) activity is directly correlated with cyclophosphamide resistance.[34,35] Whether human MSCs and pluripotent hematopoietic stem cells possess high AHD activity responsible for their 4-HC resistance remains to be established.

Previous studies on murine experimental models have shown a subclinical residual MSC damage after in vivo administration of high-dose cyclophosphamide in multiple courses (500 mg/kg × five courses).[36-38] On the other hand, addition of irradiation to lower doses of cyclophosphamide (1,500 rad + 160 mg/kg × four courses) produced a persistent MSC damage in the treated animals, suggesting that radiation may be the main factor affecting the MSC function after sequential radiation and cyclophosphamide.[39] In humans, there is good clinical evidence that very high doses of cyclophosphamide administered alone in vivo do not produce irreversible damage to the MSCs. In fact, a number of patients with small-cell lung carcinoma[28] and aplastic anemia have shown recovery of autologous bone marrow function after cyclophosphamide in maximally tolerated doses (ie, higher doses cause fatal cardiac necrosis).[40-43] Our data, in accordance with these in vivo observations, indicate that doses of 4-HC currently used in vitro for autologous human marrow transplantation have a minor quantitative toxic effect on MSCs with no significant functional damage.

The data presented in this paper and the observation that hematopoietic reconstitution occurs after the infusion of CFU-GM–depleted, 4-HC–treated marrow grafts, indicate that pluripotential stem cells (which appear not to be represented by CFU-Mix) and MSCs are resistant to 4-HC treatment up to 300 μmol/L. In addition, the generation of LTMCs from 4-HC–treated marrows represents an appropriate means for examining the toxicity of this as well as other agents on MSC populations. The regeneration of committed stem cells in this Dexter-type LTMC system may be a better indicator of the survival of an earlier pluripotent hematopoietic stem cell than the recovery of hematopoietic colony-forming cells.

REFERENCES

1. Takamizawa A, Matsumoto S, Iwata T, Katagiri K, Tochino Y, Yamaguchi K: Studies on cyclophosphamide metabolites and their related compounds. II. Preparation of an active species of cyclophosphamide and some related compounds. J Am Chem Soc 95:985, 1973
2. Fenselau C, Kan M-NN, Rao SS, Myles A, Friedman OM, Colvin M: Identification of aldophosphamide as a metabolite of cyclophosphamide in vitro and in vivo in humans. Cancer Res 37:2538, 1977
3. Sharkis SJ, Santos GW, Colvin M: Elimination of acute myelogenous leukemic cells from marrow and tumor suspensions in the rat with 4-hydroperoxycyclophosphamide. Blood 55:521, 1980
4. Kaizer H, Stuart RK, Brookmeyer R, Colvin M, Santos GW: Autologous bone marrow transplantation in acute leukemia: A phase I study of in vitro treatment of marrow with 4-hydroperoxycyclophosphamide to purge tumor cells. Blood 62:224a, 1983 (abstr)
5. Gulati S, Gandola L, Vega R, Yopp J, Chang TT, Ibrahim S, Siena S, Castro-Malaspina H, Colvin M, Clarkson B: Chemopurification of bone marrow in vitro and its clinical application. Proc Am Assoc Cancer Res 25:201, 1984 (abstr)
6. Wolf NS: The haematopoietic microenvironment. Clin Haematol 8:469, 1979
7. Dexter TM: Stromal cell associated hemopoiesis. J Cell Physiol [Suppl] 1:87, 1982
8. Piovella F, Nalli G, Malamani GD, Majolino I, Frassoni F, Sitar GM, Ruggeri A, Dell'Orbo C, Ascari E: The ultrastructural localization of factor VIII-antigen in human platelets, megakaryocytes and endothelial cells utilizing a ferritin-labeled antibody. Br J Haematol 39:209, 1978
9. Beckstead JH, Bainton DF: Enzyme histochemistry of bone marrow biopsies: Reactions useful in the differential diagnosis of leukemia and lymphoma applied to 2-micron plastic sections. Blood 55:386, 1980
10. Burgio VL, Magrini U, Ciardelli L, Pezzoni G: An enzyme histochemistry approach to the study of the human bone-marrow stroma. Acta Haematol (Basel) 71:73, 1984
11. Keating A, Singer JW, Killen PD, Striker GE, Salo AC, Sanders J, Thomas ED, Torning D, Fialkow PJ: Donor origin of the in vitro hemopoietic microenvironment after marrow transplantation in man. Nature 298:280, 1982
12. Dexter TM: Is the marrow stroma transplantable? Nature 298:222, 1982
13. Fauser AA, Messner HA: Identification of megakaryocytes, macrophages and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 78:1023, 1979
14. Lu L, Broxmeyer HE, Meyers PA, Moore MAS, Thaler HT: Association of cell cycle expression of Ia-like antigenic determinants on normal human multipotential (CFU-GEMM) and erythroid (BFU-E) progenitor cells with regulation in vitro by acidic isoferritins. Blood 61:250, 1983
15. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiareri D, McKenzie S, Broxmeyer HE, Moore MAS: Characterization of human bone marrow fibroblast colony forming cells (CFU-F) and their progeny. Blood 56:289, 1980
16. Castro-Malaspina H, Ebell W, Wang SY: Human bone marrow fibroblast colony-forming units (CFU-F), in Berk PD, Castro-Malaspina H, Wasserman LR (eds): Myelofibrosis and the Biology of Connective Tissue. Prog Clin Biol Res 154:209, 1984
17. Moore MAS, Sheridan AP: Pluripotential stem cell replication in continuous human, prosimian, and murine bone marrow. Blood Cells 5:297, 1979
18. Moore MAS, Broxmeyer HE, Sheridan APC, Meyers PA, Jacobsen N, Winchester RJ: Continuous human bone marrow culture: Ia antigen characterization of probable pluripotential stem cells. Blood 55:602, 1980
19. Gartner S, Kaplan HS: Long term culture of human bone marrow cells. Proc Natl Acad Sci USA 77:4756, 1980

20. Pelus LM, Saletan S, Silver RT, Moore MAS: Expression of Ia-antigens on normal chronic myeloid leukemic human granulocyte-macrophage colony forming cells (CFU-GM) is associated with the regulation of cell proliferation by prostaglandin E. Blood 59:284, 1982

21. Castro-Malaspina H, Saletan S, Gay RE, Oettgen B, Gay S, Moore MAS: Immunocytochemical identification of cells comprising the adherent layer of long-term human bone marrow cultures. Blood 58:107a, 1981 (abstr)

22. Lillie RD, Ashburn LL: Supersaturated solutions of fat stains in dilute isopropanol for demonstration of acute fatty degeneration not shown by Herxeheimer's technique. Arch Pathol 36:432, 1943

23. Korbling M, Hess AD, Tutscka PJ, Kaizer H, Colvin MO, Santos GW: 4-Hydroperoxycyclophosphamide: A model for eliminating residual human tumor cells and T-lymphocytes from the bone marrow graft. Br J Haematol 52:89, 1982

24. Messner HA, Fauser AA: Culture studies of pluripotential hemopoietic progenitors. Blut 41:327, 1980

25. Ash RC, Detrick RA, Zanjani ED: Studies of human pluripotential hemopoietic stem cells (CFU-GEMM) in vitro. Blood 58:309, 1981

26. Humphries RK, Eaves AC, Eaves CJ: Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. Proc Natl Acad Sci USA 78:3629, 1981

27. Botnick LE, Hannon EC, Vigneulle R, Hellman S: Differential effect of cytotoxic agents on hematopoietic progenitors. Cancer Res 41:2338, 1981

28. Smith IE, Evans BD, Harland SJ: High-dose cyclophosphamide (7 g/m^2) with or without autologous bone marrow rescue after conventional chemotherapy in the treatment of patients with small cell lung cancer. Cancer Treat Rev Suppl A 10:79, 1983

29. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AK, Keiliss-Borok IV: Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Transplantation 17:331, 1974

30. Kaneko S, Motomura S, Ibayashi H: Differentiation of human bone marrow-derived fibroblastoid colony forming cells (CFU-F) and their roles in hemopoiesis in vitro. Br J Haematol 51:217, 1982

31. Blackburn MJ, Goldman JM: Increased haematopoietic cell survival in vitro induced by a human marrow fibroblast factor. Br J Haematol 48:117, 1981

32. Blackburn MJ, Goldman JM: Increased survival of haematopoietic progenitor cells in vitro induced by human marrow stromal cells, in Baum SJ, Ledney GD, Khan A (eds): Experimental Hematology Today. Basel, Kargerm, 1981, p 77

33. Gordon MY, Kearney L, Hibbin JA: Effects of human marrow stromal cells on proliferation by human granulocytic (GM-CFC), erythroid (BFU-E) and mixed (Mix-CFC) colony forming cells. Br J Haematol 53:317, 1983

34. Hilton J, Cohen D: Role of aldehyde dehydrogenase in the response to L1210 cells to cyclophosphamide. Proc Am Assoc Cancer Res 23:169, 1983 (abstr)

35. Hilton J, Colvin M: The role of aldehyde dehydrogenase activity in cyclophosphamide sensitivity of hematopoietic and leukemic cell populations. Proc Am Assoc Cancer Res 25:339, 1984 (abstr)

36. Fried W, Husseini S, Gregory S, Knospe WH, Trobaugh FE: Effect of cyclophosphamide on the hematopoietic microenvironmental factors which influence hematopoietic stem cell proliferation. Cell Tissue Kinet 6:155, 1973

37. Fried W, Kedo A, Barone J: Effects of cyclophosphamide and of busulphan on spleen colony-forming units and on hematopoietic stroma. Cancer Res 37:1205, 1977

38. Fried W, Barone J: Residual marrow damage following therapy with cyclophosphamide. Exp Hematol 8:610, 1980

39. Wathen LM, Knapp SA, De Gowin RL: Suppression of marrow stromal cells and microenvironmental damage following sequential radiation and cyclophosphamide. Int J Radiat Biol 7:935, 1981

40. Baran DT, Griner PF, Klemperer MR: Recovery from aplastic anemia after treatment with cyclophosphamide. N Engl J Med 295:1522, 1976

41. Territo MC for the UCLA Bone Marrow Transplantation Team: Autologous bone marrow repopulation following high dose cyclophosphamide and allogeneic marrow transplantation in aplastic anemia. Br J Haematol 36:305, 1977

42. Sensebrenner LL, Steele AA, Santos GW: Recovery of hematologic competence without engraftment following attempted bone marrow transplantation in aplastic anemia. Exp Hematol 5:51, 1977

43. Gmur J, von Felten A, Rhyner K, Frick PG: Autologous hematologic recovery from aplastic anemia following high dose cyclophosphamide and HLA-matched allogeneic bone marrow transplantation. Acta Haematol (Basel) 62:20, 1979